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The role of DNA methylation in UV-induced decrease of TIMP1 and TIMP2 expressions in the human skin

사람 피부에서 자외선에 의해 감소되는 TIMP1 과 TIMP2 발현에서의 DNA 메틸화의 역할

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Abstract

The role of DNA methylation in UV-induced decrease of TIMP1 and TIMP2 expressions in the human skin

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Ultraviolet (UV) radiation poses as a harmful threat to the skin because of its mutagenic properties. It causes lesions and mutations to the base sequence of the DNA, potentially altering the vast outcome of gene expressions. Aside from UV-induced DNA damages, which has been extensively studied, UV has been reported to influence the activities of epigenetic regulation by affecting the expression of genome regulators such as DNA methyltransferases (DNMTs). DNMT1 is a "gene silencer," that is responsible for the maintenance of DNA methylation and contribution to *de novo* methylation, and DNMT3A and DNMT3B, "*de novo*" methyltransferases, harness the ability to create new methylation patterns. In UV-irradiated skin, the levels of matrix metalloproteinases (MMPs) has been reported to elevate, and the levels of tissue inhibitor of metalloproteinases (TIMPs), an inhibitor of MMPs, to decrease. In this study, we examined the role of DNMT1 in the suppression of TIMP1 and TIMP2 in UV-irradiated human skin. We observed an increase in DNMT1 expression in a time-dependent manner through *in vivo* and *in vitro* experimentations using acutely UV-irradiated human skin and UV-irradiated human dermal fibroblasts. To analyze the effect of DNMT1 on TIMP1 and TIMP2 expressions, knockdown and inhibition of DNMT1 were performed. A

decrease in DNMT1 expression resulted in an increase in TIMP1 and TIMP2. However, DNMT1 overexpression led to reduced levels of TIMP1 and TIMP2. Lastly, methylation-specific PCR results confirmed greater methylation on the CpG island residing in *TIMP2* promoter region in UV-irradiated human dermal fibroblasts. These findings suggest that UV-induced expression of DNMT1 may be responsible for mediating DNA hypermethylation in TIMP, and thus, silencing its expressions, in UV-exposed human skin.

Key words

DNA methyltransferase, ultraviolet radiation, tissue inhibitor of metalloproteinase, hypermethylation

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Introduction

Abundant in nature, ultraviolet radiation remains a determinant cause for skin senescence. Prolonged UV exposure accelerates the process of aging by degrading the structural integrity of the dermal extracellular matrix (ECM) by an overproduction of matrix metalloproteinases (MMPs) and insufficient amount of collagen synthesis (1, 2), resulting in the coarse and wrinkled appearance of the skin, which is typical of photoaged skin. MMPs constitute a large number of proteinases, each with the capacity to degrade every dermal ECM protein. Past reports elucidated elevated levels of MMP1 (interstitial collagenase), MMP3 (stormelysin-1), and MMP9 (gelatinase) following UV irradiation (3-5). Regulatory actions to counterbalance the change in MMP expressions have been reported to precede at the level of transcription, translation, proenzyme activation, and endocytosis (6). The activity of MMPs are antagonized by endogenous matrix metalloproteinase inhibitors, termed tissue inhibitor of metalloproteinases (TIMPs) (7).

Four members of TIMPs (TIMP1, TIMP2, TIMP3, and TIMP4) have been identified. In relation to the skin, a study indicated expressions of all four TIMPs to be localized mainly in the human skin dermis (1). They have a broad spectrum of overlapping specificities and affinities for MMP. Studies revealed that TIMP1 is capable of inhibiting most MMPs, except MMP19 (8). Its interaction with the active form of MMP1 and 3 and pro-MMP9, in particular, has been evidenced to form a reversible non-covalent enzyme-inhibitor complex, hence rendering the enzyme inactive (6, 9). TIMP2 has also been

reported to interact with most MMPs, but particularly, with MMP2 and MMP9 (10). Besides their MMP inhibitory activities, TIMPs possess other biological capabilities involving erythroid-potentiating activity, growth promoting effects, anti-tumoral, anti-apoptotic, and anti-angiogenic effects (10, 11).

In photoaged and intrinsically aged skin, TIMP1 has been reported to decrease (12). We speculated DNA methylation to be a possible explanation for decreased TIMP expression by UV. This process involves the addition of a methyl group to the C5 side chain of a cytosine that precede a guanine in the DNA sequence, also known as a "CpG" dinucleotide by DNA methyltransferases (DNMTs) (13). There are two types of DNMTs, of which DNMT1 is regarded the principal enzyme. DNMT1 is classified as a "maintenance" DNMT because of its role in preserving original methylation patterns, and DNMT3A and DNMT3B, "*de novo*" methyltransferases, for creating new methylation patterns (13). Another component of the DNA methylation machinery are the methyl-cytosine binding domain proteins (MBDs). MBDs acts as an aid to DNMTs by coordinating the recruitment of histone deacetylases (HDACs) to the specific site of methylated DNA, resulting in chromatin condensation and transcriptional silencing (14, 15).

To date, many studies have reported observing significant aberrant methylation patterns in TIMPs in various types of cancer cells and have identified DNMT to be the source of instigation (10, 16, 17). However, little has been reported on the events of DNA methylations accounting for modified expressions of TIMP in UV-irradiated human skin. Therefore, the objective of this study was to examine whether DNA methylation is responsible for the

decrease in TIMP1 and TIMP2 expressions in the human skin in response to acute UV irradiation. We verified altered expressions of DNMT1 and MBD1 through *in vivo* and *in vitro* experimentations using acutely UV-irradiated human skin and UV-irradiated human dermal fibroblasts, and by modulating DNMT1 expressions, we confirmed the effect of DNMT1 on TIMP1 and TIMP2 expression. Lastly, methylation-specific PCR on *TIMP2* revealed greater methylation in response to UV irradiation. These findings suggest that UV-induced DNMT1 may be responsible for mediating DNA hypermethylation in TIMP1 and TIMP2 expressions in the human skin.

Materials and Methods

Human skin samples

Nine human participants, without current or prior skin diseases, provided skin samples. A Waldmann UV-800 (Waldmann, Villingen-Schwenningen, German) phototherapy device with a F75/85W/UV21 fluorescent sun lamp, with an emission spectrum between 285 and 350 nm (peak at 310-315 nm) was used as the source of UV light. A Kodacel filter (TA401/407) (Rochester, NY) was mounted 2 cm in front of the UV tube to remove UVC of wavelengths less than 290 nm. Every individual was subjected to 2 minimal erythema dose (MED) of UV. Non-irradiated and irradiated buttock skin specimens were obtained by punch biopsy 24h after UV-irradiation. All procedures involving human subjects received prior approval from Seoul National University Institutional Review Board, and all subjects provided written informed consent. The study was conducted according to the Declaration of Helinski Principles.

Cell culture

Following informed consent, primary human dermal fibroblasts were isolated from foreskin specimens of healthy male donors of age 10 to 30 and cultured in DMEM (Gibco, Rockville, MD) with glutamine (2 mM), penicillin (400 U/mL), streptomycin (50 mg/mL), and 10% FBS (Gibco) in a humidified 5% CO_2 atmosphere at 37°C. Cultured human dermal fibroblasts (HDFs) at passages 6–10 were used for the experiments. This study was approved by the

institutional review boards of Seoul National University Hospital and conducted according to the Declaration of Helsinki principles.

Chemical and reagents

The inhibitor 5-Aza-2'-deoxycitidine, (5aza-dC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal anti-DNMT1 antibody was purchased from Merck Millipore (Darmstadt, Germany), monoclonal anti-DNMT3a and monoclonal anti-DNMT3b antibodies from Novus Biologicals, LLC (Littleton, CO, USA), monoclonal anti-MBD1 antibody, monoclonal anti-MeCP2 antibody, monoclonal anti-TIMP1 and β-actin from Santa Cruz Biotechnology (Santa Cruz, California, USA), and monoclonal anti-TIMP2 from NeoMarkers (Fremont, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin solution, penicillin/streptomycin (400 U/mL, 50 g/L) from Gibco (Carlsbad, CA, USA).

UV irradiation and 5aza-dC treatment

Primary human dermal fibroblasts of passages 10-15 were seeded at $2x10^5$ cells/well in 35mm culture dishes and grown in DMEM containing 10% FBS and subconfluent cells were incubated for 24h in serum-free media followed by another 24h in growth media. Media was collected and washed with PBS, and 1ml of PBS was added to each dish and irradiated at 100 mJ/cm^2 with a UV source from Philips TL20W/12RS fluorescent sun lamp (Einthoven, Netherlands) with an emission spectrum between 275 and 380 nm. Kodacel

filter TA401/407 from Kodak (Rochester, NY) was used to block UVC of wavelength below 290 nm. The UV irradiation intensity was measured with a Model 585100 UV meter from Waldmann (Villingen-Schwenningen, Germany). After irradiation, PBS was aspirated and 1ml of growth media containing 10% FBS was added back to each dish for 48h post-UV for the measurement of protein expression and mRNA accumulation, respectively.

A total of $1x10^5$ primary human dermal fibroblasts/well of passages 10-15 were seeded in 35mm culture dishes and grown in DMEM containing 10% FBS until the cells reached 70% confluence. Growth media was replaced with 1ml of starvation media for 24h and 1ml of growth media containing 10% FBS for another 24h. 5-aza-dC, an inhibitor of DNMT1, purchased from Sigma-Aldrich, was diluted in dimethyl sulfoxide (DMSO) and dissolved in 1ml of DMEM containing 10% FBS to reveal the appropriate final concentrations of 0, 2.5, 5, 7.5, and 10uM. Cells were treated with 5aza-dC for 48h, followed by the analysis of protein and mRNA measurements. For the protocol concerning UV-irradiation of 5aza-dC treated cells, the aforementioned UV irradiation procedure was applied to the 5aza-dC treated cells, but instead of adding 1ml of DMEM containing 10% FBS, 1ml of 5aza-dC diluted media was given post UV-irradiation.

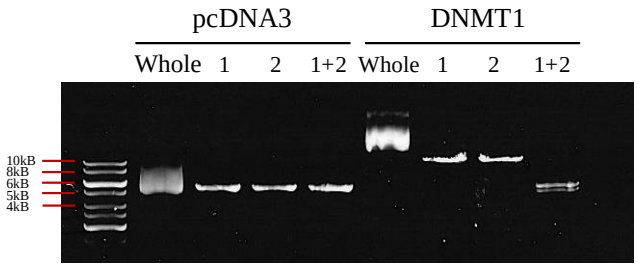
siRNA Transfection

Gene silencing of DNMT1 was performed by transiently transfecting the cells with the following siRNAs: Accutarget negative control siRNA from Bioneer (Daejeon, S. Korea) for scrambled control and DNMT1 siRNA, sense-

5’GACUACGCGAGAUUCGAGUdTdT-3’, antisense 5’-ACUCGAAUCUCGCGUAGUCdTdT-3’. When primary human dermal fibroblasts reached 50% confluence on a 35mm culture dish, the appropriate siRNAs (150pM) were transfected using the G-fectin Kit from Genolution (Daejeon, S. Korea) and incubated for 48 hours until cell harvest.

Restriction-enzyme digestion of plasmid vector

To verify the identity of the plasmid, the size of pcDNA3 and pcDNA3 Myc-DNMT1 plasmids were measured on 0.8% agarose gel proceeding incubation with restriction enzymes, Not1 and EcoR1, at 37ºC for 3 hours. Restriction enzymes were used to make (1) a one-cut to verify the entire length of the plasmid and (2) a two-cut to measure the length of only the DNMT1 insert without pcDNA3.



Restriction enzymes
1: Not1
2: EcoR1
1+2: Not1 + EcoR1

pcDNA3
1cut: 5446 bp
2cut: 5446 bp
pcDNA Myc-DNMT1
1cut: 10,297 bp
2cut without insert: 4880 bp & 5417 bp

Transfection of plasmid vector

Overexpression of DNMT1 was performed by transiently transfecting 50% confluent human dermal fibroblasts with 2.5ug of each, pcDNA3 and pcDNA3 Myc-DNMT1 plasmid, purchased from Addgene (Cambridge, MA, USA), using Lipofectamine 3000 from Thermo Fisher Scientific (Fair Lawn, NJ, USA), for 6 hours. Media was freshly changed to DMEM containing 10% FBS until the cells reached 90% confluence.

Western Blotting

Western analysis was performed by extracting whole cells and lysing the cells with RIPA lysis buffer 1x from Intron Biotechnology (Gyeonggi-do, S. Korea) mixed with protease inhibitor cocktail and phosphatase inhibitor cocktail purchased from Sigma-Aldrich (St. Louis, MO, USA). The total cell extract protein concentration was quantified via the Bradford assay, purchased from Bio-rad (Hercules, CA, USA). Equal amounts of protein, 30ug, of the lysates were subject to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then blotted to PVDF membranes. After blocking for 1h in 5% non-fat milk diluted with Tris-buffered saline containing 0.4% Tween 20 (TBST), the membrane was incubated overnight with primary antibodies (1:1000) in 4°C. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1h at room temperature. Immunoreactive bands were detected using ECL Substrate from Pierce (Rockford, IL, USA).

Immunofluorescence staining

For immunofluorescence staining, human skin tissues, fixed in 10% formalin for 24h, were embedded in paraffin wax for 4um thick sectioning. Following the standard procedure, the tissue was blocked for 30min at room temperature using a blocking solution from Zymed (San Francisco, CA, USA) and incubated overnight at 4°C using the following primary antibodies: monoclonal anti-DNMT1 from Cell Signaling (Danvers, MA, USA) 1:50 TRS pH 6.0, monoclonal anti-DNMT3b from Novus Biologicals, LLC (Littleton, CO, USA) 1:200 TRS pH 6.0, and polyclonal anti-DNMT3a from Santa Cruz Biotechnology (Santa Cruz, California, USA) 1:100 TRS pH 9.0. Bound primary antibodies were detected with streptavidin conjugated Alexa 488 from Invitrogen (Oregon, USA). Nuclei were counterstained with 4',6-diamidino-2-pheylindole (DAPI). Immunofluorescence images were taken with a Zeiss LSM 510 Laser Scanning microscope.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from primary human dermal fibroblasts using RNAiso Plus from Takara Bio Inc. (Shiga, Japan), and then reverse transcribed into cDNAs using the First Strand cDNA Synthesis Kit from MBI Fermentas (Vilnius, Lithuania) according to the manufacturer's instructions. cDNAs were subjected to amplification reactions with 7500 Real Time PCR system from Applied Biosystems (Foster City, CA) and CYBR Premix Ex Taq II Kit from Takara Bio. 2-ΔΔCt was used to calculate the fold change in the expression

level of the target gene from the threshold cycle values, which were normalized to 36B4. Primer sequence information can be found in Supplementary Table 1.

Genomic DNA extraction

Whole cell extracts were collected with PBS from 100 mm culture dishes, transferred into Eppendorf tubes, and centrifuged at 7,500 rpm for 10 mins. The supernatant is carefully removed without disturbing the cell pellet. The samples are stored at -70°C or -20°C until further processing. DNA from cultured cells were extracted using a AccuPrep Genomic DNA Extraction Kit from Bioneer (Daejeon, S. Korea) using the protocol as recommended by the manufacturer. A final volume of 50ul was used for the elution buffer.

Bisulfite conversion and methyl-specific PCR (MSP)

To determine the extent of hypermethylation at CpG islands in target gene promoter sequences, bisulfite conversion using the BisulFlash DNA Modification Kit from Epigentek (Farmingdale, NY, USA) was performed to modify unmethylated cytosine residues into uracil and to retain methylated cytosine residues. Conseqeuently, the sodium bisulfite treated DNA of the methylated and the unmethylated would differ and be distinguishable by methyl-specific PCR primers designed to have complementary sequences to the formerly unmethylated and methylated genome.

The primer sequences for the MSP primers can be found in Supplementary Table 2. DNA amplification was performed using reagents prepared by Methylamp MS-PCR Fast Kit from Epigentek (Farmingdale, NY,

USA). The thermal profile consisted of 95°C for 7 min as the initial denaturation step, 45 repetitive cycles of 95°C for 10 sec, 58°C for 10 sec, 72°C for 8 sec for DNA amplification, followed by a final extension step of 72°C for 1 min. PCR products were analyzed using agarose gel electrophoresis and GelGreen staining.

Statistical analysis

All statistical analysis was performed using Microsoft Excel 2015 software and IBM SPSS Statistics. To compare the statistical difference between two independent groups, Mann-Whitney U Test was used, and to compare the statistical differences between two or more groups, Kruskal-Wallis Test was used. A P-value of 0.05 was considered significant. All data are represented as means $\pm$ SEM.

Table 1. Primer sequences of human genes for quantitative real-time PCR

Human Gene	Forward	Reverse
h36B4	TCGACAATGGCAGCATCTAC	TGATGCAACAGTTGGGTAGC
hDNMT1	GCCCTGCATGCGGGACCT	GCCGCCGCTTAAAGGCGTTC
hDNMT3b	GGAGAAAGCTAGGGTGCGAG	ATTTCCTACTGFCTGCACGA
hDNMT3a	CCTGTGGGAGCCTCAATGTT	CCACACACTCCACGCAAAAG
hMBD1	CGCTCCCAAGGGTCTCACTGC	AGTGCCTACCACAGGCCAGGT

Table 2. Primer sequences of human genes for methylation specific PCR (MSP)

Human Gene	Forward	Reverse
hTIMP2	GGTTTTTGTTTTAAAGGATATTTTTTG	ACTCCTTACCTACATCTACATTACA
hTIMP2-U	TTTGGTGTTTTGGAAGAATGGGTG	CCAACCCCAATCCCCACTACA
hTIMP2-M	TTTGGTGTTTTGGAAGAACGGGCG	CGACCCCGATCCCCGCTACG

Results

DNMT1 and MBD1 expressions were increased in acutely UV-irradiated human skin *in vivo*

To investigate the effect of UV irradiation on methylation in the human skin *in vivo*, protein and mRNA levels of DNA methylation-associated genes were observed in sham- and in acutely UV-irradiated whole buttock skin tissue biopsied 24h post-UV (Figure 1a and 1b). In contrast to *de novo* methyltransferases, DNMT3A and DNMT3B, the maintenance DNMT, DNMT1, expression was significantly heightened. Methyl-CpG binding domain 1 (MBD1), in correspondence, also exhibited an increase.

DNMT3B, in conjunction with DNMT1, was regarded mandatory components in implementing transcriptional silencing (18). For a closer inspection of the temporal expression profile of DNMT1 and DNMT3B, immunofluorescence staining was performed in the sham-irradiated and UV-irradiated buttock skin biopsied 24, 48, and 72h post-UV (Figure 1c). Given UV exposure, the expression of DNMT1 markedly increased in a time-dependent manner in comparison to the sham-irradiated control in the epidermis and the dermis. In contrast, the change in DNMT3B expression by UV were indiscernible in both the epidermis and the dermis.

Figure 1

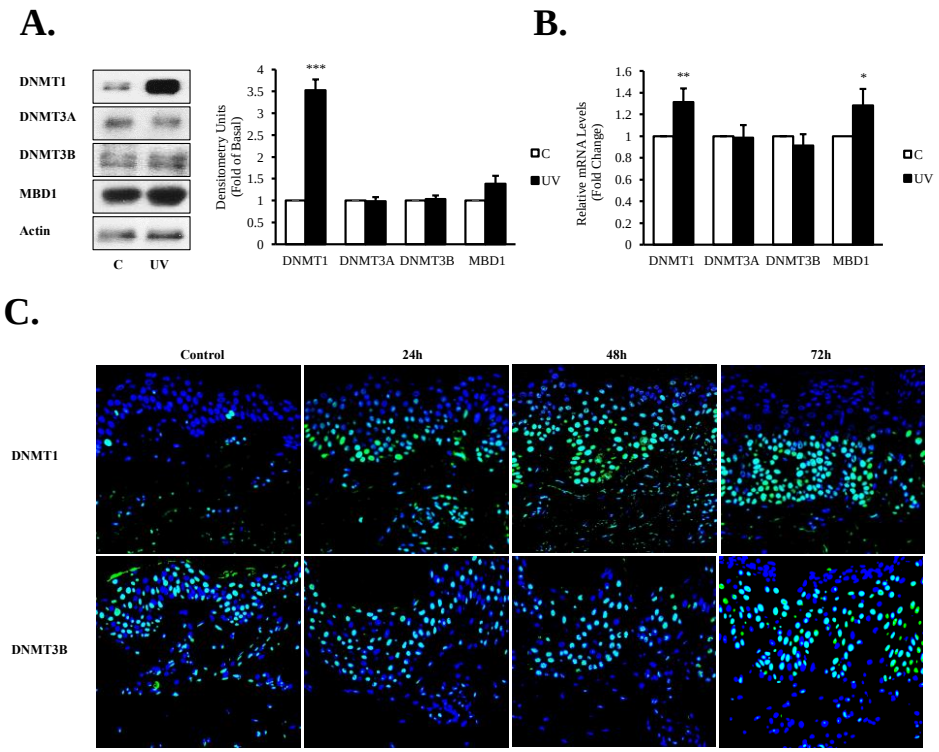

A.
DNMT1
DNMT3A
DNMT3B
MBD1
Actin
C
UV
Densitometry Units (Fold of Basal)
***
DNMT1
DNMT3A
DNMT3B
MBD1
C
UV
B.
Relative mRNA Levels (Fold Change)
**
*
DNMT1
DNMT3A
DNMT3B
MBD1
C
UV
C.
Control
24h
48h
72h
DNMT1
DNMT3B

Figure 1. Expressions of DNMT1, DNMT3A and DNMT3B, and MBD1 in acutely UV-irradiated human skin *in vivo*

Human buttock skin that was acutely irradiated with 2 MED of UV exposure was biopsied at 24 hours post-UV and subjected to (A) analysis of DNMT1, DNMT3A, DNMT3B, and MBD1 protein levels by Western blotting and the relative unit of densitometry was measured. Data represent mean ± SE of relative expressions (n = 9). Statistical comparison was made using Mann-Whitney U Test. ***P<0.001 versus sham-irradiated control. (B) mRNA levels were analyzed by quantitative real-time PCR. Data represent mean ± SE of relative expressions (n = 9). Statistical comparison was made using Mann-Whitney U Test. *P<0.05, **P<0.01 versus sham-irradiated control. Tissue samples collected at the time points of 24, 48, and 72 hours were subjected to (C) immunohistological staining of DNMT1 and DNMT3B expressions using the paraffin-embedded sections (n = 3).

DNMT1 expression was increased by UV irradiation in human dermal fibroblasts *in vitro*

To confirm the effect of UV irradiation on DNMT1 induction in human dermal fibroblasts, cells were irradiated with 100 mJ/cm^2 of UV and harvested at 24, 48, and 72h post-UV for analysis. In agreement with the *in vivo* results, an increase was seen in the protein level of DNMT1 at 48 and 72h post-UV (Figure 2a). Congruent results were found for mRNA level of DNMT1 which demonstrated an increase at an earlier time at 24 and 48h post-UV (Figure 2b).

Figure 2

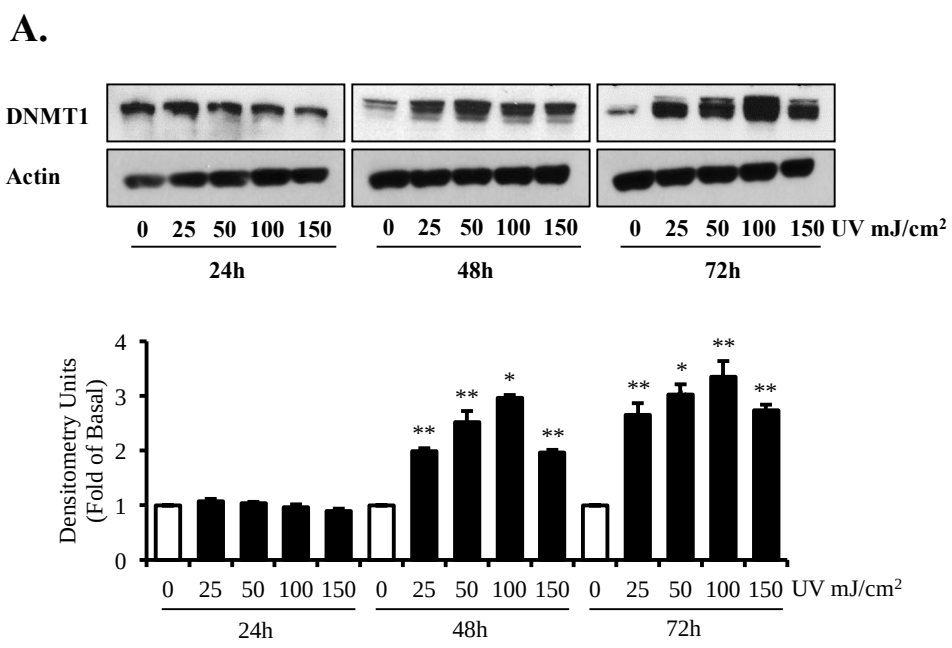

A.
DNMT1
Actin
0 25 50 100 150 0 25 50 100 150 0 25 50 100 150 UV mJ/cm2
24h
48h
72h
Densitometry Units (Fold of Basal)
0 1 2 3 4
0 25 50 100 150 0 25 50 100 150 0 25 50 100 150 UV mJ/cm2
24h
48h
72h
B.
Relative mRNA Level (Fold Change)
0 0.5 1 1.5 2
0 25 50 100 150 0 25 50 100 150 0 25 50 100 150 UV mJ/cm2
24h
48h
72h

Figure 2. DNMT1 expression in UV-irradiated human dermal fibroblasts *in vitro*

Human dermal fibroblasts were exposed to varying doses of UV and harvested at time points of 24, 48, and 72 hours. (A) DNMT1 protein expression was analyzed by subjecting cell lysates to Western Blotting and its relative unit of densitometry was quantified. Data represent mean ± SE of relative expressions (n = 7). Statistical comparison was made using Mann-Whitney U Test. **P<0.01 versus non-irradiated control. (B) The mRNA level of DNMT1 was measured by quantitative real-time PCR. Data represent mean ± SE of relative expressions (n = 7). Statistical comparison was made using Mann-Whitney U Test. *P<0.05, **P<0.01 versus non-irradiated control.

DNMT1 and MBD1, but not DNMT3A and DNMT3B, were induced by UV irradiation in human dermal fibroblasts

To assess whether congruent results as *in vivo* can be obtained *in vitro*, human dermal fibroblasts were UV-irradiated at 100 mJ/cm^2 and harvested at 48h post-UV. In agreement with our *in vivo* findings, DNMT1 displayed a significant rise in mRNA and protein levels (Figure 3a and 3b). MBD1 also showed significant increases for both mRNA and protein levels. However, no change was detected for *de novo* methyltransferases, DNMT3A and DNMT3B.

Figure 3

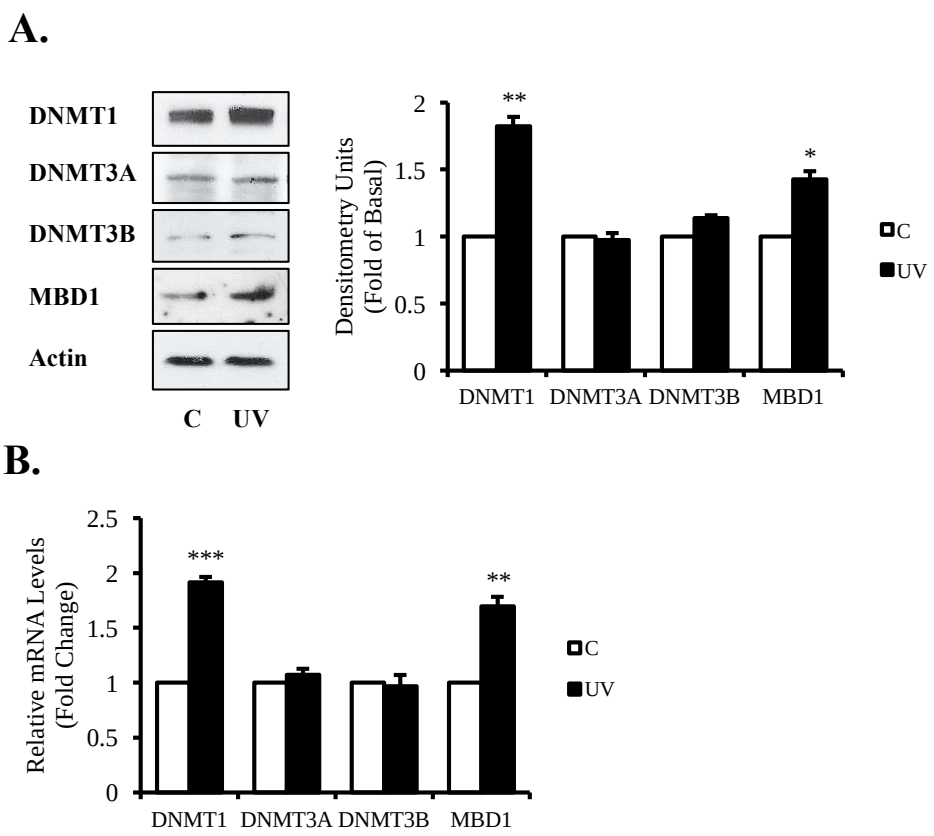

A.
DNMT1
DNMT3A
DNMT3B
MBD1
Actin
C
UV
Densitometry Units (Fold of Basal)
2
1.5
1
0.5
0
**
*
DNMT1
DNMT3A
DNMT3B
MBD1
C
UV
B.
Relative mRNA Levels (Fold Change)
2.5
2
1.5
1
0.5
0
***
**
DNMT1
DNMT3A
DNMT3B
MBD1
C
UV

Figure 3. Expressions of DNMT1, DNMT3A and DNMT3B, and MBD1 in UV-irradiated human dermal fibroblasts

Human dermal fibroblasts that were exposed to UV (100 mJ/cm^2) were harvested 24 hours after UV irradiation for (A) measurement of protein levels of DNMT1, DNMT3A, DNMT3B, and MBD1 by Western Blotting, quantification of band densitometry and (B) measurement of mRNA levels of DNMT1, DNMT3A, DNMT3B, and MBD1 by quantitative real-time PCR. Data represent mean ± SE of relative expressions (n = 5). Statistical comparison was made using Mann-Whitney U Test. *P<0.05, **P<0.001, ***P<0.001 versus non-irradiated control.

TIMP1 and TIMP2 expressions were reduced by UV irradiation in the human skin and in human dermal fibroblasts

To assess the change in TIMP1 and TIMP2 expression as a response to UV irradiation, we analyzed its expressions in acutely UV-irradiated human skin *in vivo* and in UV-irradiated human dermal fibroblasts *in vitro.* In UV-irradiated human skin, TIMP1 and TIMP2 were significantly decreased in UV-irradiated human skin when compared to the sham-irradiated control skin (Figure 4a). To confirm this result, cultured human dermal fibroblasts were exposed to UV irradiation and harvested at 24h post-UV. *In vitro* results also showed a significant decrease in TIMP1 and TIMP2 protein (Figure 4b) and mRNA (Figure 4c) expressions. Hence, our findings show that UV irradiation reduces TIMP1 and TIMP2 expressions in the human skin.

Figure 4

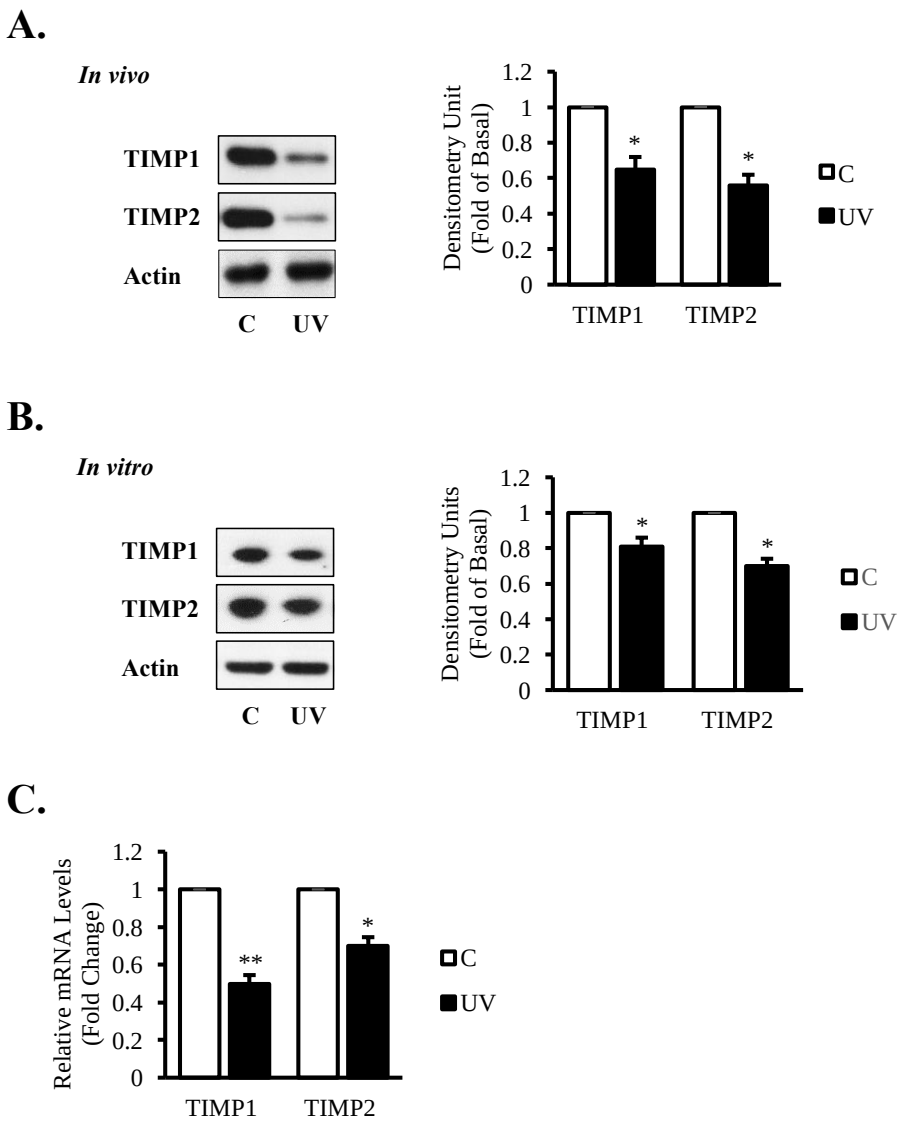

A.
In vivo
TIMP1
TIMP2
Actin
C
UV
Densitometry Unit (Fold of Basal)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
TIMP1
TIMP2
C
UV
B.
In vitro
TIMP1
TIMP2
Actin
C
UV
Densitometry Units (Fold of Basal)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
TIMP1
TIMP2
C
UV
C.
Relative mRNA Levels (Fold Change)
1.2
1
0.8
0.6
0.4
0.2
0
**
*
TIMP1
TIMP2
C
UV

Figure 4. Expressions of TIMP1 and TIMP2 in acutely UV-irradiated human skin and in UV-irradiated human dermal fibroblasts

Human buttock skin exposed to 2 MED of UV and biopsied 24 hours after UV irradiation was subjected to measurement of (A) TIMP1 and TIMP2 protein expressions using Western Blotting and its relative unit of densitometry. Data represent mean ± SE of relative expressions (n = 5). Statistical comparison was made using Mann-Whitney U Test. *P<0.05 versus sham-irradiated control. Human dermal fibroblasts irradiated with UV (100 mJ/cm^2) and harvested after 48 hours were subjected to measurement of TIMP1 and TIMP2 (B) protein levels by Western Blotting, relative unit of densitometry, and (C) mRNA levels by quantitative real-time PCR. Data represent mean ± SE of relative expressions (n = 5). Statistical comparison was made using Mann-Whitney U Test. *P<0.05, **P<0.01 versus non-irradiated control.

Knockdown of DNMT1 upregulated UV-induced decrease of TIMP1 and TIMP2 expressions in human dermal fibroblasts

Contingent on the account that DNMT1 is associated with DNA hypermethylation, we hypothesized that the elevated level of DNMT1 under UV exposure leads to decreased expressions of TIMP1 and TIMP2 (19). To analyze whether UV-induced DNMT1 is associated with the decrease in TIMP1 and TIMP2 expressions, DNMT1 siRNA was transfected, and then, concomitantly UV irradiated in cultured human dermal fibroblasts. First, we confirmed that DNMT1 expression was decreased by DNMT1 siRNA transfection (Figure 5a). Measurement of TIMP1 and TIMP2 protein and mRNA expressions showed a significant increase by DNMT1 siRNA transfection when compared to the negative control-transfected, non-UV irradiated control (Figure 5b and 5c). Furthermore, TIMP1 and TIMP2 protein and mRNA expressions, which were significantly reduced by UV, were recovered with the knockdown of DNMT1 (Figure 5c).

Figure 5

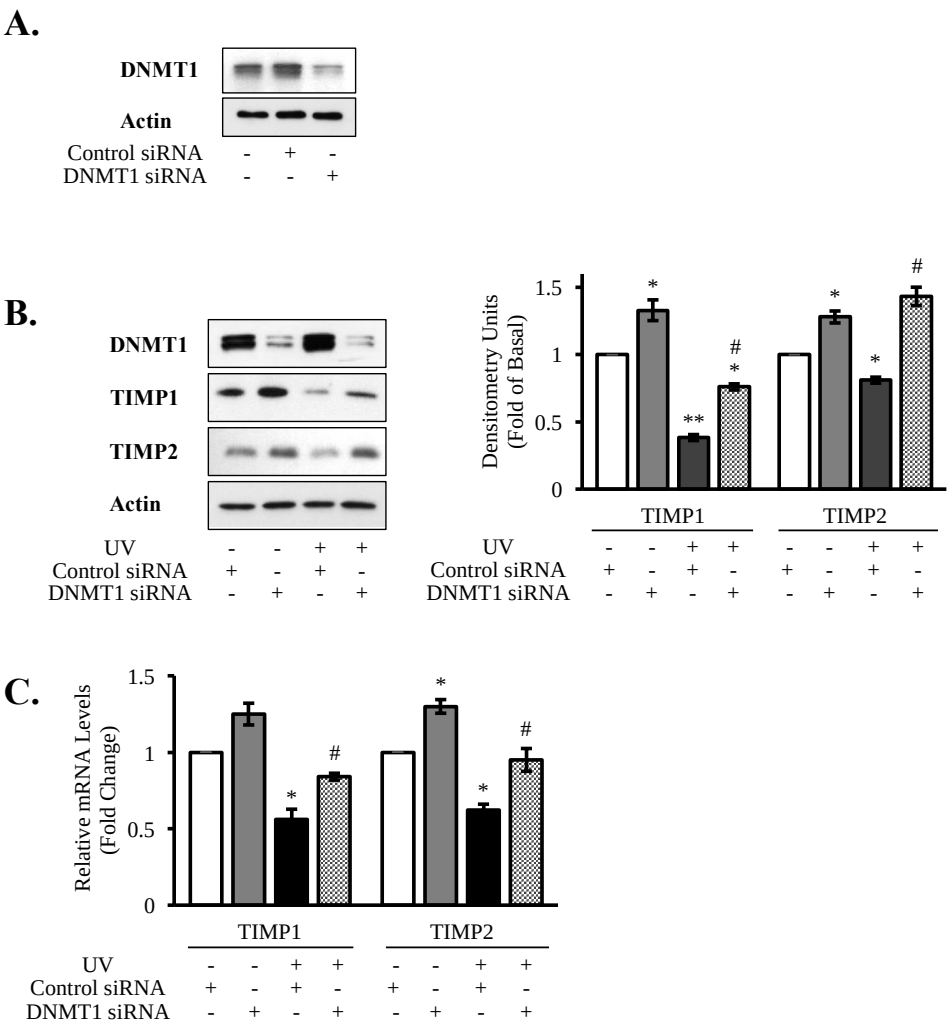

A.
DNMT1
Actin
Control siRNA - + -
DNMT1 siRNA - - +
B.
DNMT1
TIMP1
TIMP2
Actin
UV - - + +
Control siRNA + - + -
DNMT1 siRNA - + - +
Densitometry Units (Fold of Basal)
1.5
1
0.5
0
TIMP1
TIMP2
C.
Relative mRNA Levels (Fold Change)
1.5
1
0.5
0
TIMP1
TIMP2
UV - - + + - - + +
Control siRNA + - + - + - + -
DNMT1 siRNA - + - + - + - +

Figure 5. Expression of TIMP1 and TIMP2 in DNMT1 siRNA transfected human dermal fibroblasts

Human dermal fibroblasts were subjected to DNMT1 siRNA transfection for 48 hours and harvested for analysis of (A) DNMT1 protein levels by Western Blotting to confirm transfection efficiency. Subsequently, human dermal fibroblasts were transfected with scrambled control or DNMT1 siRNAs for 48 hours prior to serum starvation for 24 hours, treatment with 10% FBS for 24 hours, and harvest at 48 hours post-UV irradiation (100 mJ/cm^2). (B) Protein expressions of DNMT1, TIMP1, and TIMP2 were measured by Western Blotting, relative unit of densitometry quantified, and (C) mRNA levels measured by quantitative real-time PCR. Data represent mean $\pm$ SE of relative expressions (n = 5). Statistical comparison was made using Kruskal-Wallis Test. *P<0.05, **P<0.01 versus non-irradiated, scramble siRNA-transfected control; #P<0.05 versus irradiated, scramble siRNA-transfected control.

Inhibition of DNMT1 increased TIMP1 and TIMP2 expressions in human dermal fibroblasts

Consistent findings were seen with the inhibition of DNMT1 using 5aza-dC in cultured human dermal fibroblasts. Treatment of 5aza-dC in various doses exhibited increasing protein expressions of TIMP1 and TIMP2 in conjunction with decreasing protein expressions of DNMT1 in a dose-dependent manner (Figure 6a). Concomitant treatment of 5aza-dC with UV exposure showed a significant recovery, respectively, in UV-induced suppression of TIMP1 and TIMP2 protein and mRNA expressions (Figure 6b and 6c). These results indicate that the inhibition of DNMT1 increases TIMP1 and TIMP2 expression.

Figure 6

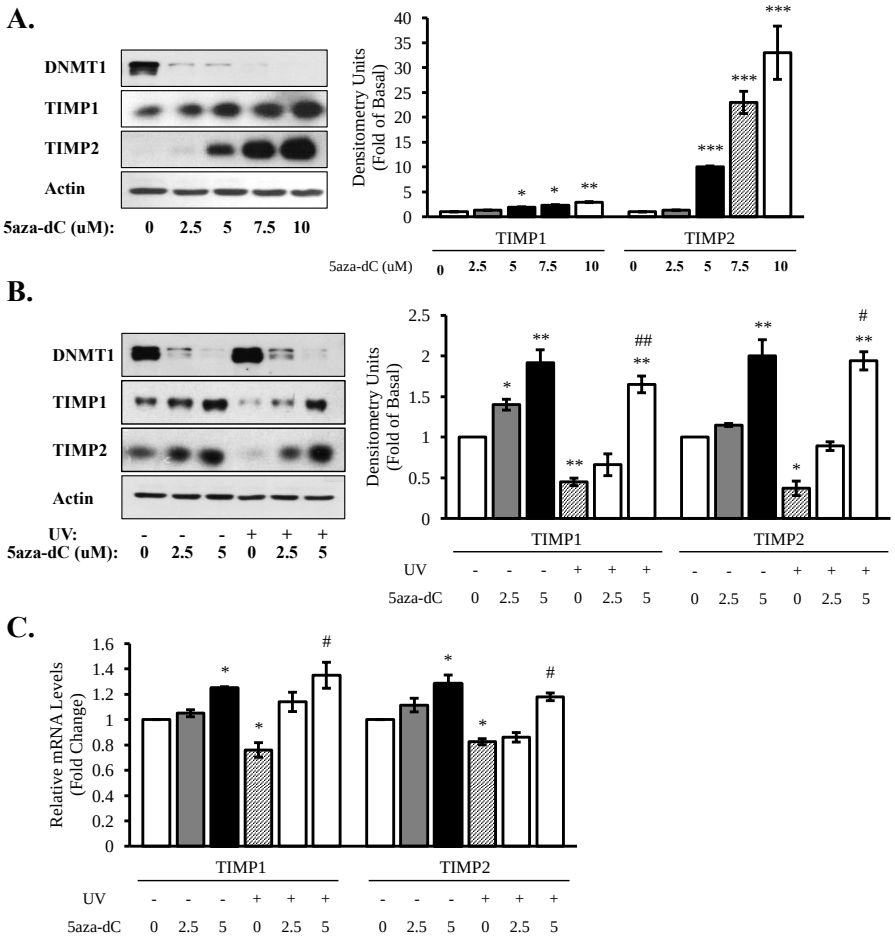

A.
DNMT1
TIMP1
TIMP2
Actin
5aza-dC (uM): 0 2.5 5 7.5 10
Densitometry Units (Fold of Basal)
TIMP1
TIMP2
5aza-dC (uM) 0 2.5 5 7.5 10 0 2.5 5 7.5 10
B.
DNMT1
TIMP1
TIMP2
Actin
UV: - - - + + +
5aza-dC (uM): 0 2.5 5 0 2.5 5
Densitometry Units (Fold of Basal)
TIMP1
TIMP2
UV - - - + + + - - - + + +
5aza-dC 0 2.5 5 0 2.5 5 0 2.5 5 0 2.5 5
C.
Relative mRNA Levels (Fold Change)
TIMP1
TIMP2
UV - - - + + + - - - + + +
5aza-dC 0 2.5 5 0 2.5 5 0 2.5 5 0 2.5 5

Figure 6. Expression of TIMP1 and TIMP2 in 5aza-dC, DNMT1 inhibitor, treated human dermal fibroblasts

To confirm our findings, human dermal fibroblasts were treated with varying doses of 5aza-dC for 48 hours under the same condition as above and subjected (A) protein measurement of DNMT1, TIMP1, and TIMP2 by Western Blotting (n = 3). The relative unit of densitometry was measured. Data represent mean ± SE of relative expressions (n = 3). Statistical comparison was made using Mann-Whitney U Test. *P<0.05, **P<0.01, ***P<0.001 versus vehicle-treated control. With the addition of UV (100 mJ/cm^2) to 5aza-dC treated cells, (B) protein levels were measured by Western Blotting, its relative unit of densitometry measured, and (C) mRNA levels quantified by quantitative real-time PCR. Data represent mean ± SE of relative expressions (n = 5). Statistical comparison was made using Kruskal-Wallis Test. *P<0.05, **P<0.01 versus non-irradiated, vehicle-treated control; #P<0.05, #P<0.01 versus irradiated, vehicle-treated control.

DNMT1 downregulated TIMP1 and TIMP2 protein expressions in human dermal fibroblasts

To further confirm that UV-induced DNMT1 mediates hypermethylation of TIMP1 and TIMP2, resulting in their decreased mRNA and protein expressions, analysis by overexpression of DNMT1 in human dermal fibroblasts was performed. Detection of Myc-Tag confirmed transfection of Myc-Tagged DNMT1 plasmid (Figure 7). Conforming to our previous results, TIMP1 and TIMP2 expressions were decreased upon DNMT1 overexpression. Our findings are suggestive of DNMT1's involvement in the decrease of TIMP1 and TIMP2 expression by UV exposure.

Figure 7

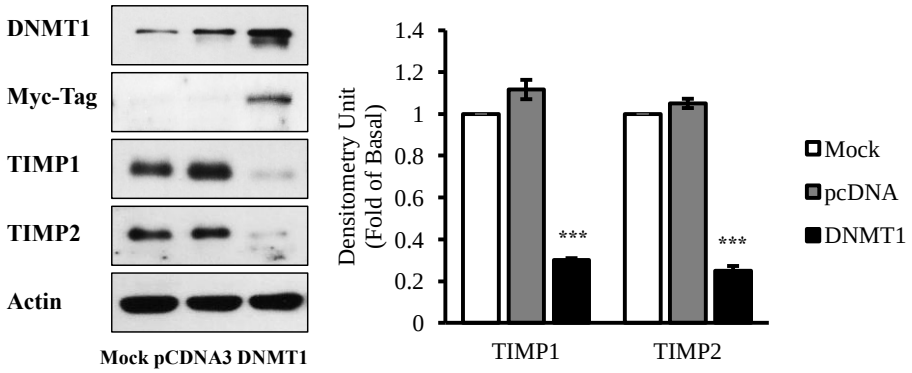


Figure 7. Effect of DNMT1 overexpression on TIMP1 and TIMP2 in human dermal fibroblasts

Human dermal fibroblasts were transfected with DNMT1 plasmid DNA for 12 hours and harvested 48 hours after. Cell lysates were analyzed for TIMP1 and TIMP2 expressions using Western Blotting. Myc-Tag was used to verify effective transfection of Myc-DNMT1 plasmid. Data represent mean ± SE of relative expressions (n = 3). Statistical comparison was made using Mann Whitney U Test. ***P<0.001 versus pcDNA3-transfected control.

DNA methylation increased at the CpG island of *TIMP2* promoter by UV irradiation

Since we postulated that the increased DNMT1 expression by UV may mediate DNA methylation in *TIMP2,* we examined the change in DNA methylation in *TIMP2* promoter after UV-irradiation by methylation-specific PCR. A schematic representation of the *TIMP2* promoter region (approximately ~900bp of length) portrays one large CpG island, from which the MSP-specific primer -896/-731, consisting of 15 CpG sites, was selected (Figure 8a). Non-irradiated control dermal fibroblasts showed partial methylation (Figure 8b). However, given UV irradiation, *TIMP2* promoter was fully methylated. In response to UV exposure, therefore, significant promoter hypermethylation was observed, which conforms to our *in vivo* and *in vitro* findings that UV-induced DNMT1 may be responsible for the hypermethylation of TIMPs.

Figure 8

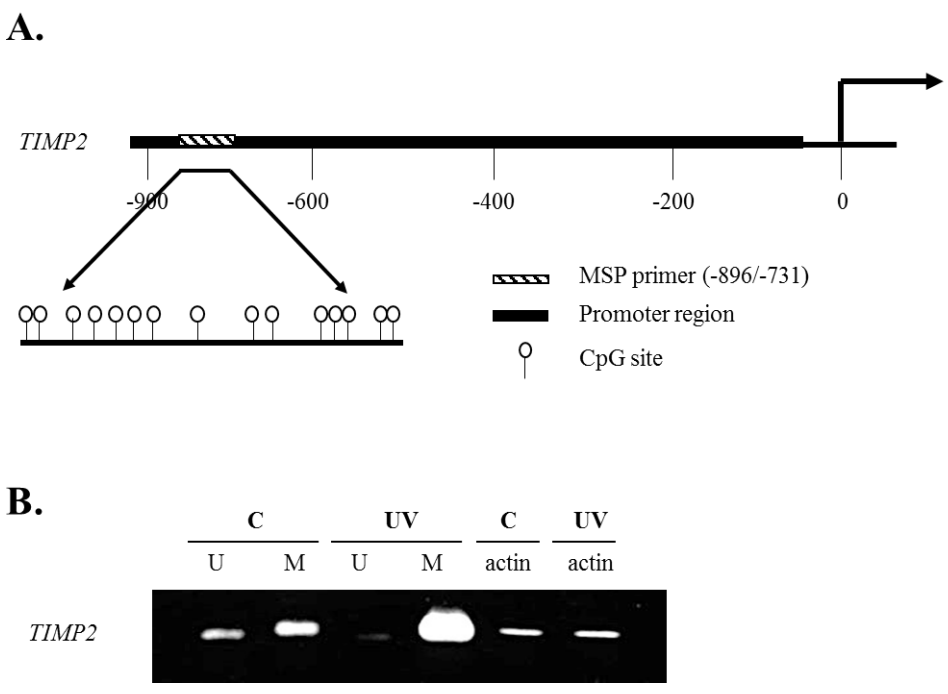

A.
TIMP2
-900
-600
-400
-200
0
MSP primer (-896/-731)
Promoter region
CpG site
B.
C
UV
C
UV
U
M
U
M
actin
actin
TIMP2

Figure 8. Methylation-specific PCR results of *TIMP2* genes in human dermal fibroblasts

(A) Schematic diagram of *TIMP2* promoter region, with indicators of the location of MSP primer, CpG island and CpG sites. (B) Methylation state of *TIMP2* was determined by methylation specific PCR *in vitro*. Human dermal fibroblasts were subjected to digestion of *EcoR1* restriction enzyme for 3 hours at 37°C, standard PCR amplification of the region of interest using TIMP2 primer, bisulfite modification, PCR amplification using two sets of primers, one for recognizing the unmethylated sequence and the other for recognizing the methylated sequence, and electrophoresis using 2% agarose gel. U, PCR with unmethylated sequence-specific primers; M, PCR with methylated sequence-specific primers (n = 3).

Discussion

An increase in appreciation for targeted therapy through epigenetic reprogramming has escalated dramatically over the past few decades. Amongst the various mechanisms that exist within the field of epigenetics, DNA methylation has been gaining an enormous amount of interest due to convincing evidences attesting the prevalence of aberrant methylations in cancer cells (20-22). The majority of these studies, however, pertain to tumor and cancer development, and little is known about the DNA methylation changes that occur in correlation with the molecular pathways of the human skin. Our most recent study reported the importance of DNMT1 in invoking changes in the expression of *COL1A2* in UV-exposed human skin (23). However, no direct study on the effect of UV on the dynamics of DNA methylation in the human skin was performed. Hence, in this study, we analyzed UV-induced changes in DNA methylation-associated genes and demonstrated its effect on the hypermethylation of TIMP1 and TIMP2 by UV irradiation in the human skin.

Our findings demonstrated a significant increase in the protein and mRNA expression of DNMT1 and a comparative increase in the mRNA expression of MBD1 in the human skin at 24h post-UV exposure. However, those of *de novo* methyltransferases, DNMT3A and DNMT3B were not altered by UV irradiation. The most crucial process of DNA methylation is executed by DNMTs. The classical methylation model proposes that DNMT1 only preserves whereas DNMT3A and DNMT3B establishes *de novo* methylation patterns (2). New evidences claim otherwise. DNMT3B, in conjunction with

DNMT1, has been deemed compulsory in implementing transcriptional silencing (18).

Placing our focus on DNMTs, to gain a more comprehensive understanding of the change in DNMT1 and DNMT3B expressions as a response to UV over time, we performed immunofluorescence staining against DNMT1 and DNMT3B on the human skin at three varying time points of 24, 48 and 72h after UV exposure. DNMT1 showed a marked increase beginning 24h post-UV in both the epidermis and the dermis. In accordance with our protein and mRNA findings, DNMT3B revealed no visible changes at all three time points when compared to the sham-irradiated control skin. *In vitro* results using cultured human dermal fibroblasts were congruent to our *in vivo* findings, which showed elevated DNMT1 protein and mRNA expressions as a response to UV exposure.

For the global DNA methylation to rise, theoretically, DNMT3A and DNMT3B, should be at work to orchestrate new methylation patterns. However, no evident changes in their expressions were observed. Some reports have suggested that DNMT1 alone, without the assistance of *de novo* methyltransferases, DNMT3A and DNMT3B, can create an extensive degree of global and CpG island methylations (13, 19, 24, 25). Evidences supporting this notion have portrayed DNMT3A and DNMT3B as being impotent because of their preference for strong flanking sequences, which are introns that are not transcribed into RNA (26-28). Also, an analysis of the entire genome-wide methylation activity revealed DNMT1 to possess considerable *de novo* activity,

methylating repetitive and single copy sequences (25). These emerging findings suggest that although DNMT1 can singularly generate DNA methylations, the presence of other epigenetic regulatory events may have interfered with the increase in global DNA methylation. Studies on histone modifications as well as chromatin remodeling in conjunction with DNA methylation will be necessary to elucidate this matter.

On the basis that DNMT1 can mediate DNA hypermethylation, we postulated that UV-induced DNMT1 may be the cause for the decrease in TIMP1 and TIMP2 expression in UV-irradiated human skin. First, by manipulating DNMT1 expressions in human dermal fibroblasts, we elucidated DNMT1's relation with TIMP1 and TIMP2; inhibition or knockdown of DNMT1 resulted in a significant increase of TIMP1 and TIMP2 whereas overexpression of DNMT1 led to a marked decrease of TIMP1 and TIMP2**.** Thereafter, to assess the change in the degree of methylation in a CpG island within *TIMP2* promoter, MSP was performed on UV-irradiated human dermal fibroblasts. In response to UV exposure, *TIMP2* promoter became fully methylated. This finding is in accordance with previous reports that demonstrated regulation of TIMP expression by DNA methylation (16, 29, 30). Together, our findings are robust evidences that suggest DNMT1's role in generating DNA hypermethylation in TIMP1 and TIMP2 by UV irradiation in the human skin.

Aside from DNMT1, *in vivo* and *in vitro* experimentations showed increased expressions of MBD1 by UV irradiation. MBDs have been reported

to contribute to the DNA methylation machinery by recruiting DNMT1 and other histone methylation enzymes and ensuring transcriptional inactivation (31). Further inspection into the role of MBD1 and histone regulators such as HDAC and HAT in carrying out gene silencing in TIMPs would be favorable.

This study has shown that UV irradiation on the human skin decreases TIMP2 expressions through an epigenetic mechanism that may involve DNMT1-mediated DNA hypermethylation. Since TIMPs have been associated with inhibitions of matrix metalloproteinases, these findings raise possibilities for therapeutic modalities in the field of skin aging using epigenetic modulations.

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